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Seasonal variation of aflatoxin M_1 contamination in industrial and traditional Iranian dairy products

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ABSTRACT

This study aimed to determine the occurrence of aflatoxin M_1 (AFM_1) contamination in 682 dairy product samples consisting of raw milk of cow, goat and sheep; Lighvan cheese; and industrial and traditional yoghurt, Kashk and Doogh samples collected from popular markets and dairy ranches in four large Iranian cities. Thin layer chromatography (TLC) technique was used for analysis of the samples. Results showed that the incidence and levels of AFM_1 contamination in raw cow milk and industrial products (manufactured from cow milk) were higher than raw goat or sheep milk, and traditional products (made from goat and sheep milk), respectively. Moreover, seasonal variations influenced the concentration of AFM_1 in most of the analyzed dairy products. Owing to the abundance and popularity of the industrial products, contamination of these products in such a level could be a potential hazard for public health.
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1. Introduction

Aflatoxins are a group of extremely toxic metabolites of fungi produced by toxicogenic strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* in a wide variety of agricultural commodities (Mortazavi & Tabatabai, 1998). They are highly toxic, carcinogenic, mutagenic and teratogenic compounds that have been considered as causative agent in human hepatic and extra-hepatic carcinogenesis. Four common types of aflatoxins are B_1, B_2, G_1 and G_2. Among them, aflatoxin B_1 (AFB_1) is notoriously the most frequent produced mycotoxin; and has been reported to be the most powerful natural carcinogen in mammals (Creppy, 2002; Hussain, Anwar, Munawar, & Asi, 2008; Tokar & Vengušt, 2008). The International Agency for Research on Cancer (IARC, 1993) of WHO included AFB_1 as Group 1 human carcinogen.

Aflatoxin M_1 (AFM_1) is the monohydroxylated metabolite of AFB_1, metabolized by cytochrome *P450* enzyme system in liver and excreted into the milk of lactating livestock which consumed AFB_1 contaminated diet (Gürbay, Aydin, Girgin, Engin, & Şahin, 2006; Keskin, BaŞkaya, Karsli, Yurdun, & Özyaral, 2009). There is a direct relationship between the AFM_1 content in milk and AFB_1 consumption via foodstuffs. It has been estimated that about 0.5–6% of AFB_1 present in animal feed pass as AFM_1 in milk. This variability is due to different factors such as stage and order of lactation, intake level of AFB_1 and individual response (Galvano, Galofaro, & Galvano, 1996). The toxicity of AFM_1 is less than its parent compound, AFB_1; but the cytotoxic, genotoxic, and carcinogenic effects of it is well demonstrated. Hence, the IARC of WHO re-considered its carcinogenicity classification and changed it from Group 2 to Group 1 according to more recent investigations (IARC, 2002).

When dairy products are manufactured from AFM_1 contaminated milk, the toxin could be detected in them (Bakirci, 2001). Unfortunately, the content of AFM_1 remains relatively stable during processing and storage of various dairy products. This toxin cannot be inactivated by thermal processing used in dairy industry i.e. pasteurization and ultra-high-temperature (UHT) treatment (Fallah, 2010a,b).

Several countries have legislated permissible levels of AFM_1 in milk and dairy products to protect consumers specially children. These regulations are influenced by economic considerations and vary from one country to another (Fallah, 2010b). The US Food and

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Drug Administration (US FDA, 1996, p. 219) has set a limit of 0.5 μg/l for AFM_1 in milk. However, the Institute of Standards and Industrial Research of Iran (ISIRI, 2002) has accepted 0.050 μg/l as the action level for AFM_1 which is the same as European Commission permitted level (European Commission, 2001). Additionally, according to ISIRI (2002), the maximum admissible level of AFM_1 in other dairy products is 0.5 μg/kg in dried milk, 0.020 μg/kg in butter and buttermilk, 0.250 μg/kg in cheese and Kashk; and 0.050 μg/l in yoghurt and Doogh.

Several studies in the world have been undertaken to determine the occurrence of AFM_1 in milk and dairy products (Carvajal, Bolaños, Rojo, & Méndez, 2003; Elzupir & Elhussein, 2010; Ghanem & Orfi, 2009; Hampikyan, Bingol, Cetin, & Colak, 2010; Kim, Lee, Kwak, Ahn, & Jeong, 2010; Nuryono et al., 2009). However, referring to existing scientific literature, a limited number of surveys have been conducted in this field in Iran. Furthermore, no seasonal study on the levels of AFM_1 in goat milk, sheep milk and traditional dairy products has been performed in Iran. Therefore, this study aimed to determine the occurrence of AFM_1 in industrial and traditional dairy products widely marketed all over the country as well as raw milk during different seasons.

2. Materials and methods

2.1. Sample collection

A total of 682 dairy product samples composed of raw cow milk ($n = 88$), raw goat milk ($n = 65$), raw sheep milk ($n = 72$), Lighvan cheese ($n = 75$), industrial yoghurt ($n = 61$), traditional yoghurt ($n = 60$), industrial Kashk ($n = 64$), traditional Kashk ($n = 61$), industrial Doogh ($n = 71$) and traditional Doogh ($n = 65$) were obtained from dairy ranches, supermarkets and retail outlets in four large Iranian cities (Tehran, Esfahan, Tabriz and Shiraz) during year 2008. The samples were transported to the laboratory inside a digital portable refrigerator at 3 °C and stored at −20 °C until analysis for AFM_1.

2.2. Reagents

All reagents were analytically pure and purchased from Merck KgaA (Darmstadt, Germany). AFM_1 standard was obtained from Sigma Chemical Company, USA. Stock solution of AFM_1 (50 mg/ml) was prepared in a methanol/chloroform mixture (81:19, v/v) and stored at −20 °C. Before use, the stock solution was diluted with methanol/chloroform (1/1, v/v) at appropriate concentrations (Fallah, 2010a,b).

2.3. Apparatus

2.3.1. Thin layer plates

Silica gel 60 (Merck, dimensions 20 × 20 cm^2, thickness 0.2 mm). These plates were cut in 10 × 10 cm^2 dimensions and used for the experiment.

2.3.2. Ultraviolet lamp

A Fisher ultraviolet (UV) lamp with 245 and 364 nm wavelengths was obtained from Philips.

2.4. Methods

Samples of milk and dairy products were analyzed by two-dimensional thin layer chromatography (TLC) technique for presence of AFM_1 according to the method of Stubblefield (1979) and Karim (1998) as described in the previous paper (Fallah, 2010a).

A homogenized sample (50 ml or 50 g) was mixed with 10 ml of saturated sodium chloride (cooled at 4 °C) and 125 ml of chloroform (cooled at 4 °C) for 1.5 min. The mixture was transferred into a 250-ml centrifuge bottle and centrifuged at 3700 rpm, in order to separate phases. The lower phase (chloroform layer) was drained into 300-ml Erlenmeyer flask containing 5 g of anhydrous sodium sulphate and swirled intermittently for 15 min. Then the mixture was filtered through a Whatman No. 4 filter paper into a 250-ml graduated cylinder to obtain sample filtrate and the volume was recorded. The filtrate was purified by silica gel column chromatography and then submitted to solvent evaporation with a rotary vacuum evaporator until nearly dry. Subsequently, the residue was dissolved in ~4 ml of chloroform and dried by a weak stream of nitrogen at 50 °C. For AFM_1 assay, the dried extract was resuspended in 100 μl of chloroform. Thereafter, 25 μl of the sample extract and a series of 3, 6, 12 and 24 μl of standard AFM_1 working solution (0.05 μg/ml) were spotted on the thin layer plate of silica gel. The plate was developed in the first and second directions with diethyl ether:methanol:water (94:4.5:1.5, *v/v/v*) and chloroform:acetone:methanol (87:10:3, *v/v/v*), respectively. The concentration of AFM_1 in the sample was estimated by visual comparison of sample extract fluorescent spot with that of standard AFM_1 spots under long wave UV light (364 nm); using the formula discribed in the previous paper (Fallah, 2010a). The confirmatory test for AFM_1 was carried out by adding trifluoroacetic acid to the fluorescent spots and comparing the thin layer chromatographic properties of the derivatives of the sample spot and the standard. The lowest detection limit of the method is 0.0125 ppb.

2.5. Method evaluation

In order to validate our method, homogenized samples of raw milk, Lighvan cheese, yoghurt, Kashk and Doogh were spiked with AFM_1 at levels of 0.05, 0.1 and 0.2 ppb just before the test. The AFM_1 concentration was determined using the previously described procedure. The results indicated that the method was reliable for determination of AFM_1 in the mentioned dairy products (Table 1).

2.6. Statistical analyses

To evaluate seasonal effects, data obtained from each dairy product sample in different seasons were compared. Statistical analyses were carried out using one-way ANOVA of the GraphPad Prism software, version 3. The differences among means at $P < 0.05$ were compared by Tukey's Multiple Comparison Test.

Table 1
Validation of the TLC method for determination of AFM_1 in dairy products ($n = 4$).

Dairy product	Spiked level (ppb)	AFM_1 determined (ppb)	Recovery[a] (%)	Coefficient of variation (%)
Raw milk	0.05	0.045	90.5 ± 4.9	5.4
	0.10	0.088	87.6 ± 5.8	6.6
	0.20	0.175	87.5 ± 8.3	9.5
Lighvan cheese	0.05	0.040	79.8 ± 9.1	11.3
	0.10	0.088	88.5 ± 7.9	9.0
	0.20	0.175	87.3 ± 9.1	10.4
Yoghurt	0.05	0.044	87.6 ± 6.8	7.8
	0.10	0.084	83.5 ± 8.5	10.1
	0.20	0.159	79.7 ± 4.8	6.1
Kashk	0.05	0.038	75.5 ± 7.0	9.3
	0.10	0.083	82.5 ± 9.4	11.4
	0.20	0.166	83.0 ± 8.2	9.8
Doogh	0.05	0.046	92.5 ± 4.5	4.8
	0.10	0.086	85.7 ± 8.1	9.5
	0.20	0.179	89.4 ± 7.0	7.8

[a] Mean ± SD.

Table 2
Occurrence of aflatoxin M_1 in dairy products from Iran.

Dairy product	Samples analyzed, *n*	Positive samples, *n* (%)	Mean ± SEM (μg/l or μg/kg)[a]	Min–max (μg/l or μg/kg)[a]	Exceed regulation, *n* (%)[b]
Raw cow milk	88	74 (84.1)	0.052 ± 0.006	0.013–0.394	31 (35.2)
Raw goat milk	65	28 (43.1)	0.018 ± 0.003	0.013–0.055	7 (10.8)
Raw sheep milk	72	43 (59.7)	0.027 ± 0.004	0.015–0.102	18 (25.0)
Lighvan cheese	75	49 (65.3)	0.085 ± 0.010	0.030–0.313	7 (9.3)
Industrial yoghurt	61	30 (49.2)	0.026 ± 0.004	0.015–0.102	10 (16.4)
Traditional yoghurt	60	14 (23.3)	0.007 ± 0.002	0.015–0.036	0 (0.0)
Industrial Kashk	64	34 (53.1)	0.080 ± 0.013	0.028–0.285	11 (17.2)
Traditional Kashk	61	19 (31.2)	0.053 ± 0.011	0.046–0.291	1 (1.6)
Industrial Doogh	71	16 (22.5)	0.007 ± 0.002	0.013–0.053	3 (4.2)
Traditional Doogh	65	9 (13.8)	0.003 ± 0.001	0.013–0.029	0 (0.0)

[a] Results are expressed in μg/l for raw milk and Doogh; and μg/kg for cheese, yoghurt and Kashk.
[b] The ISIRI limit for aflatoxin M_1 is 0.050 ppb for milk, yoghurt and Doogh; and 0.250 ppb for cheese and Kashk.

3. Results and discussion

3.1. Raw milk

In the present study, the incidence of AFM_1 contamination in raw cow, goat and sheep milk was 84.1%, 43.1% and 59.7%; with the average values of 0.052, 0.018 and 0.027 μg/l, respectively. However, the levels of the toxin in 35.2% of cow milk, 10.8% of goat milk and 25% of sheep milk samples exceeded the ISIRI and EC limit for AFM_1 in liquid milk i.e. 0.050 μg/l (Table 2). The lower concentrations of AFM_1 in goat and sheep milk than cow milk could be due to the pasture grazing of goat and sheep in Iran. It has been demonstrated that out-pasturing is an effective factor to decrease the level of AFM_1 contamination in milk of dairy species (Hussain, Anwar, Asi, Munawar, & Kashif, 2010; Kamkar, 2005). Only for 2–3 months in winter, Iranian goat and sheep are fed by stored grains and compound feed. The obtained results are comparable to the results of previous studies performed in Iran (Ghiasian, Maghsood, Neyestani, & Mirhendi, 2007; Kamkar, 2005; Rahimi, Bonyadian, Rafei, & Kazemeini, 2010; Tajkarimi et al., 2008); but are higher than those reported in European countries (Bilandžić, Varenina, & Solomun, 2010; Martins & Martins, 2000; Saccà et al., 2009; Velasco, Delso, & Escudero, 2003; Virdis, Corgiolu, Scarano, Pilo, & De Santis, 2008). This is the consequence of comprehensive supervision and Hazard Analysis and Critical Control Point (HACCP) programmes developed in European countries to manage the risks associated with toxicogenic fungi contamination all along the feed supply chain; and also legislating strict regulatory limits for aflatoxins in feed and milk.

Considering seasonal effect influences, the levels of AFM_1 in cow, goat and sheep milk samples collected in winter were significantly higher ($P < 0.05$) than those collected in the other seasons (Table 3). Our findings are in agreement with previous studies that reported higher levels of AFM_1 contamination in cold seasons than hot ones (Hussain & Anwar, 2008; Nemati, Mehran, Hamed, & Masoud, 2010; Ruangwises & Ruangwises, 2010). The reason is that in cold seasons lactating animals are fed with greater amounts of compound feed which may be contaminated with higher levels of AFB_1.

3.2. Lighvan cheese

Lighvan cheese is a semi-hard, starter-free, traditional Iranian cheese produced from a mixture of raw goat and sheep milk in small dairies located in Lighvan village in East Azarbaijan province of Iran and widely consumed all over the country. The frequency of AFM_1 contamination in Lighvan cheese was 65.3%, at the mean value of 0.085 μg/kg; and 9.3% had levels of the toxin above the legal limit accepted by ISIRI i.e. 0.250 μg/kg (Table 2). Due to the preferential affinity of AFM_1 for casein fraction of milk, concentration of the toxin is higher in cheese than in milk from which the cheese is made (Ardic, Karakaya, Atasever, & Adiguzel, 2009). Referring to the existing scientific literature, no data have been published on the occurrence of AFM_1 in cheese made from goat milk, sheep milk or mixture of them in Iran. Comparing our findings with the previous studies conducted in Iran (Fallah, 2010a; Fallah, Jafari, Fallah, & Rahnama, 2009; Kamkar, 2006), this survey revealed lower AFM_1 contamination in Lighvan cheese than white cheese prepared from cow milk.

Considering seasonal variability, the mean concentration of AFM_1 in Lighvan cheese samples collected in spring was significantly higher ($P < 0.05$) than those collected in the other seasons (Table 3). Due to the duration of ripening of Lighvan cheese (3 months), most of the cheeses offered for sale in spring are manufactured from winter milk that could contain higher levels of AFM_1.

Table 3
Levels of aflatoxin M_1 in dairy products from Iran: Comparison between samples obtained in different seasons.

Dairy product	Winter		Spring		Summer		Autumn	
	Samples analyzed, *n*	Mean ± SEM (μg/l or μg/kg)[d]	Samples analyzed, *n*	Mean ± SEM (μg/l or μg/kg)[d]	Samples analyzed, *n*	Mean ± SEM (μg/l or μg/kg)[d]	Samples analyzed, *n*	Mean ± SEM (μg/l or μg/kg)[d]
Raw cow milk	24	0.093 ± 0.019[a]	21	0.031 ± 0.005[c]	21	0.028 ± 0.005[c]	22	0.051 ± 0.006[b]
Raw goat milk	18	0.035 ± 0.005[a]	17	0.013 ± 0.004[b]	16	0.011 ± 0.004[b]	14	0.012 ± 0.005[b]
Raw sheep milk	22	0.046 ± 0.008[a]	19	0.021 ± 0.007[b]	15	0.019 ± 0.005[b]	16	0.019 ± 0.006[b]
Lighvan cheese	20	0.083 ± 0.020[b]	17	0.162 ± 0.024[a]	20	0.051 ± 0.015[b]	18	0.053 ± 0.018[b]
Industrial yoghurt	16	0.038 ± 0.010[a]	15	0.013 ± 0.006[b]	16	0.012 ± 0.005[b]	14	0.041 ± 0.009[a]
Traditional yoghurt	15	0.018 ± 0.004[a]	15	0.002 ± 0.001[b]	15	0.002 ± 0.002[b]	15	0.005 ± 0.003[b]
Industrial Kashk	15	0.115 ± 0.025[a]	18	0.130 ± 0.026[a]	17	0.039 ± 0.017[b]	14	0.030 ± 0.018[b]
Traditional Kashk	15	0.047 ± 0.024	16	0.051 ± 0.022	15	0.066 ± 0.026	15	0.053 ± 0.011
Industrial Doogh	18	0.007 ± 0.004	16	0.008 ± 0.005	17	0.006 ± 0.003	20	0.009 ± 0.004
Traditional Doogh	16	0.006 ± 0.003	16	0.002 ± 0.002	17	0.000 ± 0.000	16	0.004 ± 0.002

[a,b,c]Mean ± SEM in the same row with different letters are significantly different ($P < 0.05$).
[d] Results are expressed in μg/l for raw milk and Doogh; and μg/kg for cheese, yoghurt and Kashk.

3.3. Yoghurt and yoghurt derived products

Industrial yoghurt is manufactured from cow milk in dairy industries, while traditional yoghurt is made from goat milk, sheep milk or mixture of them in ranches or small dairy shops. Our study showed that the incidence and level of AFM_1 in industrial yoghurt were higher than traditional yoghurt due to the higher occurrence of the toxin in cow milk than goat and sheep milk. The level of AFM_1 in 16.4% of the industrial samples was above the legal limit accepted by ISIRI i.e. 0.050 μg/kg; while none of the samples of traditional yoghurt exceeded the limit (Table 2). Regarding seasonal variability, the levels of the toxin in industrial yoghurt samples obtained in autumn and winter were significantly higher ($P < 0.05$) than those obtained in spring and summer. In the case of traditional yoghurt, the level of AFM_1 was significantly higher ($P < 0.05$) in winter than other seasons (Table 3).

Kashk is a special Iranian dairy product prepared by prolonged boiling of yoghurt. Traditional Kashk is a dried product with the shape of conic or cubic balls originated from goat and sheep milk. But, the industrial Kashk is a thick whitish liquid prepared from cow milk. The frequency of AFM_1 contamination in industrial and traditional Kashk was 53.1% and 31.2%, with the average values of 0.080 and 0.053 μg/kg, respectively. It was found that 11 samples (17.2%) of industrial and one sample (1.6%) of traditional Kashk had levels of the toxin in excess of the ISIRI limit i.e. 0.250 μg/kg (Table 2). In the case of industrial Kashk, winter and spring levels of AFM_1 contamination were significantly higher ($P < 0.05$) than summer and autumn levels of the toxin. In contrast, no statistically significant seasonal effect ($P > 0.05$) was observed for traditional Kashk samples (Table 3).

Doogh is a traditional Iranian fermented product prepared from yoghurt by adding potable water, starter culture and sodium chloride. Iranian Doogh is exported and consumed in Afghanistan, Azerbaijan, Armenia, Turkey, Balkans and to a lesser extent in other parts of Middle East and central Asia (Codex Alimentarius Commission, 2009). In the present study the incidence of AFM_1 contamination in Doogh samples was approximately low, since the toxin was detected in 22.5% of industrial Doogh (manufactured from cow milk) and 13.8% of traditional Doogh samples (prepared from goat and sheep milk). The average values of AFM_1 contamination were 0.007 and 0.003 μg/l in industrial and traditional Doogh, respectively; and only 4.2% of the industrial Doogh samples exceeded the ISIRI limit i.e. 0.050 μg/l (Table 2). No statistically significant seasonal effect ($P > 0.05$) was found for Doogh samples obtained in different seasons (Table 3).

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